AVIAN DISEASES 53:552–562, 2009

Biologic Characterization of H4, H6, and H9 Type Low Pathogenicity Avian Influenza Viruses from Wild Birds in Chickens and Turkeys

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Received 30 April 2009; Accepted and published ahead of print 28 July 2009

SUMMARY. The pathogenesis, virus shedding, and serologic response in specific-pathogen-free (SPF) chickens and commercial turkeys against H4, H6, and H9 type low pathogenic avian influenza viruses (LPAI) from wild birds was examined. Four-week-old chickens and three-week-old turkeys were given 1×10^6 EID_{50} of LPAI per bird, intrachoanally, and examined for clinical signs for 3 wk. Oropharyngeal and cloacal swabs, and fecal samples, were collected at 2, 4, and 7 days postinoculation (PI) for virus detection by real-time RT-PCR. Serum was collected at 7, 14, and 21 days PI and examined for antibodies against avian influenza virus (AIV) by the enzyme-linked immunosorbant assay (ELISA) and hemagglutination inhibition tests. Tissue samples for histopathology were collected from three birds per group at 3 days PI. The hemagglutinin genes of the viruses were sequenced, and phylogenetic analysis was conducted. Clinical signs ranged from no clinical signs to moderate depression, decreased activity, and decreased food and water consumption. Based on virus detection results, SPF chickens were generally found to be shedding more virus from both the oropharynx and cloaca than were commercial turkeys. Microscopic lesion results in both species showed the predominance of lesions in the respiratory and gastrointestinal tract, which is consistent with the fact that these viruses are of low pathogenicity. In chickens and turkeys, oropharyngeal shedding strongly correlated with the lesions found in the upper respiratory tract. Turkeys had fewer lesions in the respiratory tract and more lesions in the gastrointestinal tract compared to chickens. Thirteen LPAI viruses caused seroconversion in commercial turkeys, whereas only 6 LPAI viruses caused seroconversion in SPF chickens. Phylogenetic analysis of the HA genes showed that the H4, H6, and H9 viruses evaluated here represented the full genetic diversity of North American AIVs of their respective subtypes. This data is important for surveillance and control because some of the LPAI viruses (of wild bird origin and examined in this study) that can infect and be shed by chickens and turkeys would be difficult to detect in commercial poultry. Specifically, detection is difficult because these viruses did not cause overt clinical disease or mortality, but only induced mild microscopic lesions and exhibited poor seroconversion.

RESUMEN. Caracterización biológica en pollos y pavos de los virus de la influenza aviar de baja patogenicidad subtipos H4, H6 y H9 detectados en aves silvestres.

Se evaluó en pollos libres de patógenos específicos y en pavos, la patogénesis, la eliminación viral y la respuesta serológica contra virus de la influenza aviar de baja patogenicidad subtipos H4, H6, y H9 que fueron aislados de aves silvestres. Pollos de cuatro semanas y pavos de tres semanas de edad fueron inoculados directamente en las coanas con virus de la influenza aviar de baja patogenicidad con una dosis por ave de 1×10^6 dosis infectantes para embrión de $pollo_{50\%}$ (EID_{50}) y se examinaron en sus signos clínicos por tres semanas. Se recolectaron muestras de hisopos orofaríngeos y cloacales a los dos, cuatro, y siete días postinoculación para la detección por RT-PCR en tiempo real. Se recolectaron muestras para suero a los siete, catorce y 21 días después de la inoculación y se analizaron para detectar anticuerpos contra el virus de la influenza aviar mediante un ensayo de inmunoabsorción con enzimas ligadas (ELISA) y por la prueba de inhibición de la hemoaglutinación. Se recolectaron muestras de tejidos para histopatología de tres aves por grupo a los tres días después de la inoculación. Se secuenciaron los genes de la hemaglutinina de los virus estudiados y se llevó a cabo el análisis filogenético. La signología clínica incluyó desde la ausencia de signos clínicos a depresión moderada, disminución de la actividad y disminución en el consumo de alimento y agua. Con base en los resultados de detección, los pollos libres de patógenos específicos mostraron mayor eliminación viral en la orofaríngе y en cloaca en comparación con los pavos comerciales. Los resultados del estudio de las lesiones microscópicas en ambas especies mostró la predominancia de lesiones en los tractos respiratorio y gastrointestinal, que fueron consistentes con los virus de baja patogenicidad. En pollos y pavos, la eliminación orofaríngea estuvo fuertemente correlacionada con las lesiones encontradas en el tracto respiratorio superior. Los pavos presentaron menos lesiones en el tracto respiratorio y mayores lesiones en el tracto gastrointestinal en comparación con los pollos. Trece de los virus de la influenza de baja patogenicidad causaron seroconversión en pavos comerciales, mientras que solo seis virus de baja patogenicidad causaron seroconversión en pollos libres de patógenos específicos. El análisis filogenético de los genes HA mostró que los virus H4, H6 y H9 evaluados en este estudio representaron completamente a la diversidad genética observada con los virus de la influenza en Norteamérica y sus subtipos respectivos. Estos datos son importantes para los sistemas de vigilancia y el control porque algunos de los virus de la influenza aviar de baja patogenicidad (con origen en aves silvestres examinados en este estudio) que pueden infectar y pueden ser eliminados por pollos y pavos pudieran ser difíciles de detectar en la avicultura comercial. Específicamente, la detección es difícil porque estos virus no causan una enfermedad clínica evidente o mortalidad, sino solo inducen lesiones microscópicas leves y mostraron una seroconversión baja.

Key words: avian influenza virus, biological characterization, low pathogenicity, wild birds

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Abbreviations: AI = avian influenza; AIV = avian influenza virus; BALT = bronchial associated lymphoid tissue; BSL = biosafety level; C = cloacal; CT = cycle threshold; EID_{50} = 50% embryo infectious dose titer; ELISA = enzyme-linked immunosorbant assay; F = fecal; GALT = gut associated lymphoid tissue; HA = hemagglutination; HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; LPAI = low pathogenicity avian influenza; LRE = linear regression equation; NVSL = National Veterinary Services Laboratory; OP = oropharyngeal; PBS = phosphate-buffered saline; PI = postinoculation; RT-PCR = reverse transcriptase-polymerase chain reaction; SPF = specific-pathogen-free

Avian influenza viruses (AIV) continue to be a problem worldwide because they are potentially highly infectious, can rapidly spread and cause disease in domestic poultry, and some viruses may also infect other animal hosts, including humans. Around the globe, an enormous number of poultry have died from direct infection with AIV, and countless numbers of poultry flocks at risk have been depopulated as a measure to contain the virus and prevent its further spread (6). Apart from the severe economic losses in commercial poultry, AIV can evolve rapidly and cross into other species (8,18,31). More recently, AI viruses have been isolated from several animal species, some of which include highly pathogenic avian influenza (HPAI) H5N1 viruses infecting wild felids (tigers and leopards [*Panthera* spp.]), domestic cats (*Felis catus*), dogs (*Canis lupus*), and humans (12,14,21).

Charadriiform (shorebirds and gulls) and anseriform (ducks, geese and swans) birds are known to be the natural hosts of AIV. These wild aquatic birds usually do not show clinical signs of disease following infection with AIV (24). In addition, HPAI viruses in domestic poultry are thought to have evolved from low pathogenic wild bird influenza viruses through mutations or recombination (3). As many of these wild birds fly great distances, they can interact with other species, including domestic poultry, and play a key role in the transmission, evolution, and pathogenesis of the virus. However, a long-standing debate still exists as to the exact role of wild birds in the spread of AIV among domestic poultry, with the current focus on HPAI H5N1-type viruses because of their zoonotic potential (1,18). In addition, other subtypes of the virus, particularly the H4-, H6-, and H9-type viruses, are prevalent in wild birds and may infect and cause clinical disease directly in poultry as well as serving as a source of genes for reassortment with H5 and H7 viruses (3,10,16).

The implications of a waterfowl and shorebird role in the introduction of AIVs to poultry, the spread of AIVs within poultry populations, the prevalence of low pathogenic avian influenza (LPAI) viruses in wild birds, and the potential for H5 and H7 LPAI viruses to mutate and produce HPAI in chickens, warrant the biologic characterization of those LPAI viruses from wild birds in domestic avian species. However, little has been done to biologically characterize other important hemagglutinin types of AIV from wild birds in domestic avian species. The H4 and H6 virus infections commonly occur in ducks worldwide, and they are routinely isolated from shorebirds at Delaware Bay (7,24). The H9 viruses are underrepresented in wild bird isolates, but have been documented in ducks, gulls, and shorebirds (7,11,13,24), and an H9N2 lineage is currently associated with domestic poultry infections in the Middle East and Asia (9).

In this study, we biologically characterized H4, H6, and H9 type LPAI virus wild bird isolates in chickens and turkeys. We examined the pathogenesis, virus shedding, and serologic response in specific-pathogen-free (SPF) chickens and commercial turkeys against these virus isolates. These data provide insights toward further understanding of the ecology of avian influenza viruses and contributes information that could lead to the development and implementation of strategies for the prevention and control of avian influenza in domestic poultry and, ultimately, in humans.

MATERIALS AND METHODS

Viruses and virus titrations. Sixteen LPAI wild bird virus isolates used in this study (Table 1) were obtained from Dr. David Stallknecht (Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA). Viruses of each subtype were selected for diversity in date of isolation, location of origin, and species of origin. Viruses were passaged twice and then titrated in 10-day-old SPF embryonating eggs to determine the 50% embryo infectious dose titer (EID_{50}) calculated by the Reed and Muench method (32). Lesions found in embryos on the last day of a 7-day incubation were recorded, and the hemagglutination (HA) test was performed on the allantoic fluid of all surviving embryos (27). In addition, the HA test was used to determine the HA titer of each virus examined in this study.

Birds and facilities. Four-week-old SPF white leghorn chickens (Merial Select, Gainesville, GA) and 3-wk-old commercial turkeys (Sleepy Creek Hatchery, Goldsboro, NC) were used in this study. A minimum of eight birds per group were utilized. All experiments were conducted in a biosafety level (BSL) 2 Ag+ facility at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, University of Georgia, (Athens, GA) in accordance with USDA-APHIS Permit Number 103372. The facility consists of a stand-alone block building with controlled access. The building has an anti-room that leads to the main room where the isolators are maintained. Stainless steel and polycarbonate, negative pressure, and HEPA-filtered isolator units with internal dimensions of 42″L × 24″W × 31″ H were used to house individual groups of birds. Each isolator unit had its own air-handling equipment, a 2-door pass-through access port, a radiant heater, height-adjustable feeders, nipple drinkers, and a sealed collection canister for manure.

Experimental design. Viruses were diluted with sterile phosphate-buffered saline (PBS, pH 7.4) to adjust the amount of inoculum to 1×10^6 EID_{50} per 0.1 ml per bird. For viruses below 1×10^6 EID_{50} per 0.1 ml concentration, 0.1 ml of undiluted virus per bird was given. So that these data can eventually be compared with data from similar ongoing studies with different AIV subtypes in other laboratories, the viruses were administered via the oropharyngeal (OP; intrachoanal) route. This route of inoculation has also been used in previous studies (19). For each experiment, one group of birds was not inoculated and served as negative controls. After inoculation, all birds were observed for clinical signs of disease and mortality twice daily for 21 days. Clinical signs of disease were scored and recorded as follows: 0 = no signs; 1 = mild to moderate respiratory signs; 2 = moderate respiratory signs, depressed or not eating; 3 = moderate to severe respiratory signs, depressed, not eating, and neurologic signs; and 4 = moribund birds (were removed and immediately necropsied).

Oropharyngeal and cloacal (C) swabs were collected in 1 ml of sterile PBS from each bird at 2, 4, and 7 days postinoculation (PI). Fecal samples from all isolators (including the control group) were obtained at the time of swab collection. All swab and fecal samples were stored at −80 C and thawed only once for RNA extraction. Serum was also collected from each bird at 7, 14, and 21 days PI and was stored at −20

Table 1. Low pathogenic avian influenza wild bird isolates used in the study.

Isolate	Subtype	NVSL[A] accession no.	Code	Titer (EID_{50}/ml)[B]
A/Mallard/MN/530/00	H4N6	355801	MN 00-530	$1 \times 10^{8.4}$
A/Mallard/MN/253/99	H6N5	199074	MN 99-253	$1 \times 10^{8.5}$
A/Blue-Winged Teal/LA/69B/87	H4N8	–[C]	LA 69B	$1 \times 10^{8.4}$
A/Mallard/MN/346233/2000	H6N3	346233	MN 00-38	$1 \times 10^{7.6}$
A/Ruddy Turnstone/DE/114/03	H9N2	1016395	AI 03-114	$1 \times 10^{8.4}$
A/Ruddy Turnstone/DE/1070/02	H9N4	650637	AI 02-1070	$1 \times 10^{7.4}$
A/Mallard/MN/263/99	H4N9	199076	MN 99-263	$1 \times 10^{8.7}$
A/Mallard/MN/198/99	H4N6	199059	MN 99-198	$1 \times 10^{7.5}$
A/Ring-billed Gull/GA/421733	H6N4	421733	AI 01-124	$1 \times 10^{8.5}$
A/Blue-Winged Teal/LA/B156/87	H4N6	–	LA B156	$1 \times 10^{9.2}$
A/Ruddy Turnstone/NJ/AI01-1407	H13N6	471604	AI 01-1407	$1 \times 10^{7.5}$
A/Ruddy Turnstone/NJ/452/03	H9N2	1016432	AI 03-452	$1 \times 10^{6.7}$
A/Blue-Winged Teal/LA/240B/88	H4N6	–	LA 240B	$1 \times 10^{9.9}$
A/Northern Pintail/Tx/828197/02	H6N4	828197	TX 02-260	$1 \times 10^{9.7}$
A/Mallard/MN/232/98	H9N2	182753	MN 98-232	$1 \times 10^{6.8}$
A/Ruddy Turnstone/NJ/749/02	H1N9	650616	AI 02-749	$1 \times 10^{8.1}$

[A]NVSL = National Veterinary Services Laboratory, Ames, IA.

[B]EID_{50} = Fifty percent embryo infectious dose representing the highest dilution of virus detected by embryo lesions or hemagglutination activity in allantoic fluid.

[C]A dash indicates no accession number was available from NVSL.

C. Tissue samples for histopathology were collected from three birds per group at 3 days PI.

RNA extraction and quantitative real-time RT-PCR. Viral RNA was extracted from swabs and fecal material using the MagMax-96 Total RNA Isolation kit (Ambion Inc., Austin, TX) and a KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturers' recommendations.

The Ambion Ag Path ID One Step RT-PCR kit (Ambion Inc.) was used for nucleic acid amplification with a 25-μl reaction mixture containing the following reagents: 12.5 μl of kit-supplied 2× RT-PCR buffer, 1 μl of kit-supplied 25× RT-PCR enzyme mix, and 10 μl of extracted viral RNA. Each reaction mixture utilized 10 picomoles of matrix gene primers (forward and reverse) and probe sequences (all three using 0.5 μl each) following the protocol of the real time RT-PCR assay developed for type A influenza virus (22). Real-time RT-PCR was carried out in a Smart Cycler thermocycler machine (Cepheid, Sunnyvale, CA) with the following conditions for the RT step (50 C for 30 min and 94 C for 15 min) and for the PCR cycling protocol (94 C for 15 sec and 60 C for 20 sec for 45 cycles). Data were reported as the average cycle threshold (CT) value and compared to a standard curve to determine relative amounts of virus (EID_{50}/ml) present in the samples. The standard curve was generated by extracting viral RNA from all 16 LPAI viruses inoculated in experimental birds, making 10-fold serial dilutions of these RNAs and carrying out real time RT-PCR reactions on all samples, as previously described.

Histopathology. Microscopic examination was conducted on the following tissues: heart, lung, liver, spleen, pancreas, duodenum, jejunum, cecum, cecal tonsils, ileum, bursa of Fabricius, breast and thigh muscle, thymus, nasal cavity, and brain. Tissue samples were fixed in 10% neutral-buffered formalin, routinely processed, and embedded into paraffin blocks. Thin sections were cut and stained with hematoxylin and eosin and examined by light microscopy.

Serologic testing. Sera were tested for antibodies to AIV using a commercial enzyme-linked immunosorbant assay (ELISA) kit, the FlockChek™ Avian Influenza Virus Antibody Test (IDEXX, Portland, ME). In addition, samples were tested for antibodies by the hemagglutination inhibition (HI) test using four HA units of viruses, with the highest HA titers representing H4, H6, and H9 subtypes (27).

Sequencing. The HA1 region of the influenza HA genes were amplified by RT-PCR as previously described (26). The RT-PCR products were purified by agarose gel extraction with the Qiaquick Gel Extraction kit (Qiagen, Inc., Valencia, CA) and were directly sequenced. The BigDye Terminator kit (Applied Biosystems, Foster City, CA) was used for cycle sequencing and subsequently run on an ABI 3730 sequencer (Applied Biosystems).

Phylogenetic and sequence analysis. The HA1 coding region of the HA genes (nucleotides: 1–1029 for H4, 1–1035 for H6, and 1–1014 for H9) were aligned, with selected reference isolates that represented the major clades of each subtype, with Clustal V (Lasergene, V. 8.0.2 DNAStar, Madison, WI). Trees were constructed with merged duplicate runs of BEAST v. 1.4.8 (5) using HKY substitution, empirical base frequency, Gamma heterogeneity, codon 2 partitions, relaxed lognormal clock, Yule Process tree prior with default operators with UPGMA starting tree, and an MCMC length of 10^6.

RESULTS

Virus and virus titrations. Virus titers are shown in Table 1 and ranged from $1 \times 10^{6.7}$ to $1 \times 10^{9.9}$ EID_{50}/ml. A positive correlation was observed between virus titer and severity of embryo lesions in surviving embryos. The HA titers for each virus ranged from 64 to 4096. Among all of these viruses, the three with the highest HA titers were chosen to represent H4 (LA B156, HA titer = 1024), H6 (TX 02-260, HA titer = 2048), and H9 (AI 03-452, HA titer = 1024) subtypes for the HI test.

Clinical signs and gross lesions. None of the 16 viruses caused overt clinical disease or gross lesions, except for MN 99-263 (H4N9) in turkeys, which resulted in one death and one bird with moderate respiratory signs and depression.

RNA extraction and quantitative real-time RT-PCR. Data from the C and OP swabs taken from chickens and turkeys during the experiment (days 2, 4, and 7) are shown in Table 2. Negative control birds (noninoculated) for each experimental trial were negative by quantitative real time RT-PCR (results not shown).

Among the 16 LPAI viruses inoculated into chickens, OP shedding was detected as early as day 2 PI in groups of birds infected with five different viruses (MN 00-530, LA 69B, MN 99-263, AI 01-124, and MN 99-198), while C shedding was detected in only two (AI 02-749 and MN 99-198). At day 4 PI, OP shedding was noted with eight viruses (MN 99-253, AI 03-114, LA B156, TX 02-260, AI 02-749, LA 69B, MN 99-263, and MN 99-198) while C shedding was detected in chickens inoculated with four viruses (MN 99-253, TX 02-260, AI 02-749, and LA 69B). Most of the birds

Table 2. Viral shedding summary for oropharyngeal (OP), cloacal (C), and fecal (F) samples from chickens and turkeys.

		Chicken									Turkey								
		Day 2			Day 4			Day 7			Day 2			Day 4			Day 7		
Virus	Sub-type	OP	C	F	OP	C	F	OP	C	F	OP	C	F	OP	C	F	OP	C	F
MN 00-530	H4N6	29.40 ± 3.3[A]	0	–[B]	0.00	0.00	28.88	39.21	35.94 ± 1.8	22.35	0	0	0	0	0	0	0	0	0
MN 99-253	H6N5	0	0	–	37.68	35.64	0	37.38	0	0	0	0	0	0	0	0	0	0	0
AI 03-114	H9N2	0	0	–	37.45	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA B156	H4N6	0	0	–	36.34 ± 5.1	0	0	0	38.64 ± 0.2	0	0	0	0	0	0	0	0	0	0
TX 02-260	H9N2	0	0	–	36.93 ± 0.4	36.55	0	37.31 ± 0.7	37.64	0	39.21	0	0	0	0	0	0	0	0
AI 02-749	H9N9	40.89	36.08	–	35.28 ± 0.8	36.68 ± 1.8	0	39.73 ± 1.5	0	0	0	0	0	32.91 ± 2.7	29.15	0	0	25.53	31.06
LA 69B	H4N8	30.34 ± 3.9	0	0	32.11	26.72	0	0	0	34.17	0	0	0	0	0	0	0	0	0
MN 00-38	H6N3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AI 02-1070	H9N4	38.20	0	0	0	0	0	0	0	0	38.85	0	0	0	0	0	0	0	0
MN 99-263	H4N9	32.80 ± 4.5	0	0	32.34	0	0	0	0	0	34.01 ± 0.8	0	0	0	0	0	0	0	0
AI 01-124	H6N4	32.30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MN 99-198	H4N6	29.66 ± 1.3	32.11	0	29.78	0	0	0	0	0	31.42 ± 0.4	40.97	0	36.87 ± 1.4	0	0	35.70	0	0
AI 01-1407	H4N6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AI 03-452	H9N2	0	0	0	0	0	0	0	0	0	37.24 ± 2.1	0	0	0	0	0	34.81	0	0
LA 240B	H4N6	0	0	0	0	0	0	0	0	0	35.22 ± 2.6	0	0	33.18	0	0	0	0	0
MN 98-232	H9N2	0	0	0	0	0	0	0	0	0	35.77	0	0	0	0	0	0	0	0

[A]Average CT values for the positive samples ± standard deviation (where applicable); these represent the relative amount of virus genetic material present in the sample (higher numbers = less virus). CT values of 0, and values above 38, are considered negative for the test. The number of birds examined for OP and C samples was $n = 8$ on day 2, and $n = 6$ on days 4 and 7. One representative fecal sample was collected on each day.

[B]A dash indicates no data.

Table 3. Linear regression equations (LRE) generated from standard curves of LPAI wild bird isolates.

Virus	LRE[A]	R^2 [B]	Standard error of the estimate[C]
MN 00-530	$y = -0.287x + 14.539$	0.997	0.092
MN 99-253	$y = -0.293x + 14.881$	0.993	0.143
AI 03-114	$y = -0.293x + 15.115$	0.990	0.151
LA B156	$y = -0.298x + 15.797$	0.984	0.156
TX 02-260	$y = -0.287x + 15.906$	0.982	0.203
AI 02-749	$y = -0.306x + 14.474$	0.989	0.193
LA 69B	$y = -0.315x + 16.319$	0.994	0.113
MN 00-38	$y = -0.278x + 14.580$	0.998	0.061
AI 02-1070	$y = -0.301x + 15.024$	0.950	0.263
MN 99-263	$y = -0.325x + 16.460$	0.990	0.164
AI 01-124	$y = -0.313x + 14.828$	0.979	0.242
MN 99-198	$y = -0.298x + 13.797$	0.992	0.140
AI 01-1407	$y = -0.324x + 14.558$	0.963	0.327
AI 03-452	$y = -0.252x + 12.151$	0.953	0.335
LA 240B	$y = -0.273x + 16.257$	0.990	0.135
MN 98-232	$y = -0.295x + 13.514$	0.994	0.098

[A]Dilutions used to determine LRE ranged from undiluted virus and 10^{-1} to 10^{-10}, with each dilution done in triplicate for all 16 viruses.
[B]Correlation coefficient.
[C]Standard deviation of the residuals.

were negative for OP and C shedding at day 7 PI, except for those inoculated with MN 00-530 (C), MN 99-253 (OP), and TX 02-260 (OP and C).

In turkeys, five different viruses were detected in OP swabs at day 2 PI (MN 99-263, MN 99-198, AI 03-452, LA 240B, and MN 98-232). No C shedding was detected at day 2 PI in turkeys. Virus was detected in OP and C swabs from turkeys inoculated with AI 02-749, whereas only OP shedding was detected in turkeys given MN 99-198 and LA 240B at day 4 PI. Oropharyngeal shedding was detected in turkeys given MN 99-198 and AI 03-452, while only one, AI 02-749, was detected in C swabs at day 7 PI.

Fecal samples from both species were also collected at days 2, 4, and 7 PI, and viruses were detected in samples collected from days 4 (MN 00-530 in chickens) and 7 PI (MN 00-530 and LA 69B in chickens and AI 02-749 in turkeys; Tables 2 and 4). Along with the correlation coefficients, linear regression equations (Table 3) derived from the standard curve of each virus were computed and used to determine the relative amounts of virus present in the swab and fecal samples (Table 4). Based on these results, SPF chickens were generally found to be shedding more virus from both the oropharynx and cloaca than were the commercial turkeys.

Histopathology. Microscopic lesions found in the internal organs and tissues of infected SPF chickens collected at 3 days PI are shown in Table 5. Negative control birds did not have any appreciable lesions (data not shown). Most of the lesions observed in the infected birds were predominantly confined to the respiratory and gastrointestinal tract. In the nasal cavity, 10 out of the 16 viruses caused mild to severe forms of catarrhal rhinitis, while 2 viruses (MN 99-198 and AI 03-452) caused lymphocytic rhinitis in chickens. Sloughing of the respiratory epithelium and the presence of a mild amount of mucin (catarrhal tracheitis) were noted in the tracheal lumen of chickens infected with nine different viruses, while only LA 240B-infected chickens had acute degeneration of multiple areas in the tracheal epithelium. Excessive amounts of mucin–edema in the lamina propria of a secondary bronchus (edematous bronchitis) were found in a chicken infected with MN 00-530, while a mild to moderate form of catarrhal bronchitis was noted in birds inoculated with viruses MN 98-232 and LA B156, respectively. A mild to severe form of interstitial pneumonia (MN 99-253, AI 03-114, and AI 02-749), mild to moderate proliferation of bronchiole-associated lymphoid tissues (BALT; TX 02-260 and AI 02-749), and lymphocytic bronchiolitis (LA 69B and AI 01-1407) were also found to be present in infected chickens.

A minimal amount of scattered myocardial fibers, with shrunken nuclei and loss of cross-striations (myocardial degeneration), were noted in chickens infected with MN 99-263, while all skeletal muscle samples (breast and thigh) were negative for lesions. In addition, minimal amounts of lymphocytic infiltrates were seen to surround a small blood vessel in the cerebral part of the brain (lymphocytic encephalitis) in birds infected with MN 99-263.

Lesions in the gastrointestinal tract were predominantly found in the ceca of chickens infected with five viruses, wherein mild cecal tonsil microhemorrhages and inflammation were present. Only one virus (AI 01-124) caused a focal, moderate lymphocytic infiltration in the lamina propria of the small intestine (lymphocytic enteritis). Infection with five viruses (MN 99-253, TX 02-260, AI 01-124, MN 99-198, and LA 240B) produced a mild to moderate lymphocytic hepatitis, while inoculation of AI 02-749 resulted in a mild increase in lymphocytic infiltrates in the periportal regions (lymphocytic cholangiohepatitis) of the liver. Likewise, mild hepatic necrosis was noted in liver tissues of birds infected with AI 01-1407 and LA 240B virus. A mild proliferation of gut-associated lymphoid tissues (GALT) was present in the duodenal samples of chickens infected with LA B156, AI 02-749, and MN 00-38 viruses. Also, a mild to severe form of lymphocytic pancreatitis was recorded in samples taken from birds infected with AI 02-749 and TX 02-260, respectively. Mild necrosis was observed in the spleen of a chicken inoculated with MN 00-38, while four viruses (MN 00-530, LA B156, TX 02-260, and AI 02-749) caused an increased number of macrophages throughout the parenchyma (histiocytosis).

Mild to moderate lymphoid depletion was observed in the bursa of Fabricius for seven viruses, and in the thymus for two viruses, while adrenal glands had no histologic lesions present upon examination. Mild tubular degeneration, and mild to moderate interstitial nephritis and a severe lymphocytic orchitis, was also noted in kidney and testicular sections, respectively.

Microscopic lesions for turkeys are shown in Table 6. Similar to negative controls in chickens, no significant lesions were found in noninoculated turkeys (results not shown). In the respiratory tract, viruses TX 02-260, AI 02-749, LA 69B, MN 00-38, AI 02-1070, AI 02-1070, MN 99-263, AI 01-124, AI 01-1407, LA 240B, and MN98-232 caused moderate catarrhal rhinitis and TX 02-260, MN

Table 4. Relative viral amounts[A] detected in oropharyngeal (OP), cloacal (C), and fecal (F) samples collected from chickens and turkeys.

Virus	Relative viral amount (1×10^n EID_{50}/ml)[B] in chickens									Relative viral amount (1×10^n EID_{50}/ml) in turkeys								
	Day 2			Day 4			Day 7			Day 2			Day 4			Day 7		
	OP	C	F	OP	C	F	OP	C	F	OP	C	F	OP	C	F	OP	C	F
MN 00-530	6.10	–[C]	X[D]	–	–	6.20	–	4.20	8.10	–	–	–	–	–	–	–	–	–
MN 99-253	–	–	X	3.80	4.40	–	3.90	–	–	–	–	–	–	–	–	–	–	–
AI 03-114	–	–	X	4.10	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LA B156	–	–	X	5.00	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TX 02-260	–	–	X	5.30	5.40	–	5.20	5.10	–	–	–	–	–	–	–	–	–	–
AI 02-749	–	3.40	X	3.70	3.20	–	–	–	–	–	–	–	4.40	5.50	–	–	6.70	5.00
LA 69B	6.70	–	–	6.20	7.90	–	–	–	5.50	–	–	–	–	–	–	–	–	–
MN 00-38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AI 02-1070	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MN 99-263	5.80	–	–	5.90	–	–	–	–	–	5.40	–	–	–	–	–	–	–	–
AI 01-124	4.70	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MN 99-198	5.00	4.20	–	4.90	–	–	–	–	–	4.40	–	–	2.80	–	–	3.20	–	–
AI 01-1407	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AI 03-452	–	–	–	–	–	–	–	–	–	2.80	–	–	–	–	–	3.40	–	–
LA 240B	–	–	–	–	–	–	–	–	–	6.60	–	–	7.20	–	–	–	–	–
MN 98-232	–	–	–	–	–	–	–	–	–	3.00	–	–	–	–	–	–	–	–

[A]Relative viral amounts calculated from the average CT value ($n = 8$ on day 2 and $n = 6$ on days 4 and 7) using the linear regression equations in Table 3.
[B]EID_{50} = 50% embryo infectious dose.
[C]A dash indicates no virus detected.
[D]X = denotes missing data.

99-263, and MN 98-232 caused mild proliferation of BALT, whereas MN 00-38 caused severe proliferation of BALT. Mild to moderate lymphocytic rhinitis was noted in turkeys for all of the viruses except for two (TX 02-260 and MN 99-198), with LA 69B, MN 00-38, AI 02-1070, MN 99-263, and MN 98-232 also producing mild to moderate catarrhal and heterophilic tracheitis.

Lymphocytic endocarditis was noted in the heart tissue sample of birds infected with AI 03-114, MN 00-38, AI 02-1070, and MN 98-232, while loss of cross-striations and hypereosinophilia of myofibers (degenerative myopathy) were observed in skeletal muscle samples collected from birds infected with TX 02-260, LA 69B, MN 00-38, AI 02-1070, AI 01-124, LA 240B, and MN 98-232. There were no microscopic lesions in the brain, pancreas, adrenal glands, kidneys, and reproductive organs.

Mild to moderate forms of enteritis were noted mainly in the duodenum and small intestine of turkeys infected with MN 00-530, MN 99-253, AI 03-114, LA B156, TX 02-260, LA 69B, MN 99-263, MN 99-198, AI 01-1407, LA 240B, and MN 98-232 viruses. Moreover, degeneration–necrosis of some villus tips (enteric necrosis) and multiple areas of mild heterophils in the lamina propria of the small intestine were seen in birds inoculated with AI 03-114. Proliferation of GALT was also observed in the duodenum of birds infected with MN 00-530, LA 240B, and MN 98-232. Multiple foci of mild numbers of heterophils in the lamina propria of the cecum (inflammation of the cecum or typhlitis) were found in tissues obtained from turkeys infected with AI 03-114, TX 02-260, AI 02-749, AI 01-124, AI 01-1407, LA 240B, and MN 98-232. Mild to moderate microvesicular hepatopathy was noted in liver samples taken from birds infected with MN 00-530, MN 99-253, AI 03-114, LA B156, TX 02-260, MN 99-263, AI 01-124, AI 01-1407, and MN 98-232, while specimens taken from turkeys infected with TX 02-260, AI 01-1407, and MN 98-232 showed moderate numbers of large, clear vacuoles within the cytoplasm of hepatocytes (lipidosis). TX 02-260 caused an increased number of macrophages in the splenic parenchyma, while mild lymphoid atrophy (MN 00-530, MN 99-253, LA B156, AI 02-749, AI 02-1070, MN 99-263, AI 01-124, MN 99-198, AI 01-1407, AI 03-452, LA 240B, and MN 98-232) and mild to moderate lymphoid atrophy (MN 99-253, AI 03-114, TX 02-260, AI 02-749, AI 02-1070, MN 99-263, AI 01-124, MN 99-198, AI 01-1407, AI 03-452, LA 240B, and MN 98-232) in the bursa and thymus were also noted.

Serologic testing. Although we did not test for antibodies to AIV prior to inoculation, sera from noninoculated control birds for each experimental trial were negative for antibodies to AIV in the ELISA and HI tests (results not shown). In both tests, antibodies against LPAI viruses were not demonstrated in chickens (Table 7) inoculated with 10 different viruses (MN 99-253, AI 03-114, LA B156, TX 02-260, AI 02-749, AI 02-1070, AI 01-1407, AI 03-452, LA 240B, and MN 98-232). For two viruses, MN 00-38 and AI 01-124, only one sample was positive for both tests and, in chickens given LA 69B, two birds were positive by ELISA and HI, but the titers were low (data not shown). Chickens given MN 99-198, MN 99-263, and MN 00-530 developed antibodies that were detected in both tests (Table 7).

In turkeys given viruses MN 00-38, AI 01-1407, and MN 98-232, the sera were negative for AIV-specific antibodies at all times of collection for both serologic tests (Table 7). Although the average titers were negative for turkeys given AI 03-114 and LA 69B, both groups had two positive serum samples on day 14 and one positive sample on day 21 PI. Turkeys given viruses MN 99-253, LA B156, TX 02-260, AI 02-749, AI 02-1070, MN 99-263, AI 01-124, MN

Table 5. Average severity of microscopic lesions found in body organs and tissues of LPAI virus-infected chickens ($n = 3$) at 3 days PI.

Lesion[A]	Virus[B] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Nasal cavity, trachea, and lung																
Catarrhal rhinitis	+	–	–	+	–	+/ +++	+	–	+	–	–	+	+/ ++	+	+	+/+++
Lymphocytic rhinitis	–	–	–	–	–	–	–	–	–	–	–	+	–	++	–	–
Catarrhal tracheitis	–	–	+	+	+	+	+	–	–	+	+	–	–	–	+	+
Degenerative tracheitis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Edematous bronchitis	+++	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Catarrhal bronchitis	–	–	–	++	–	–	–	–	–	–	–	–	–	–	–	+
Interstitial pneumonia	–	+	+	–	–	+++	–	–	–	–	–	–	–	–	–	–
BALT hyperplasia	–	–	–	–	+	++	–	–	–	–	–	–	–	–	–	–
Lymphocytic bronchiolitis	–	–	–	–	–	–	++	–	–	–	–	–	+	–	–	–
Heart, breast, and thigh muscle																
Myocardial degeneration	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–
Lymphocytic myocarditis	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–
Brain																
Lymphocytic encephalitis	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Gastrointestinal tract																
Cecal tonsil micro-hemorrhages	–	–	+	+	+	–	+	–	+	–	–	–	–	–	–	–
Lymphocytic enteritis	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
GALT hyperplasia	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–
Typhlitis	–	–	–	–	–	++	+	+	+	–	–	–	–	+	–	–
Liver and biliary structures																
Lymphocytic hepatitis	–	+	–	–	+	–	–	–	–	–	+	++	–	–	+	–
Lymphocytic cholangiohepatitis	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Hepatic necrosis	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–
Pancreas																
Lymphocytic pancreatitis	–	–	–	–	+++	+	–	–	–	–	–	–	–	–	–	–
Spleen																
Necrosis	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Histiocytosis	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–
Bursa and thymus																
Lymphoid depletion	+	+	–	+	+	+	–	–	–	–	–	–	++	–	+	–
Follicle atrophy	–	–	–	+	–	–	–	–	–	–	–	–	++	–	–	–
Adrenal gland	–	–	–	–	X	–	X	–	–	–	–	–	–	–	–	–
Kidney and reproductive organs																
Tubular degeneration	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Interstitial nephritis	–	–	–	–	–	–	–	–	–	+	++	+/++	–	–	–	–
Lymphocytic orchitis	–	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–

[A] – = no lesions; ± = minimal; + = mild; ++ = moderate; +++ = severe; X = no data.

[B] 1 = MN 00-530, 2 = MN 99-253, 3 = AI 03-114, 4 = LA B156, 5 = TX 02-260, 6 = AI 02-749, 7 = LA 69B, 8 = MN 00-38, 9 = AI 02-1070, 10 = MN 99-263, 11 = AI 01-124, 12 = MN 99-198, 13 = AI 01-1407, 14 = AI 03-452, 15 = LA 240B, 16 = MN 98-232.

99-198, AI 03-452, and LA 240B had positive average antibody titers in the ELISA and HI tests, but not all birds in the groups seroconverted. By day 21, all of the turkeys given MN 00-530 seroconverted. Based on this data, 13 LPAI viruses caused seroconversion in commercial turkeys, whereas only six LPAI viruses caused seroconversion in SPF chickens.

HA gene sequence analysis. Two viruses, which had been initially identified as an H9 (AI 02-749) and an H4 (AI 01-1407) by the HI assay, were shown to be H1 and H13, respectively, when sequence data was obtained. The H1 subtype virus (AI 02-749) was from the North American wild bird H1 lineage, which is distinct from both the swine and human H1 lineages. The H13 virus (AI 01-1407) was most closely related to shorebird H13s isolated in the United States in the late 1980s; it is distinct from European-origin H13s.

The H4, H6, and H9 viruses were initially selected to represent diversity within their respective subtypes—when sequence information was available. The HA1 regions of the H4 isolates had between 91.1% and 96.6% identity, the H6 isolates had between 69.5% and 99.4% identity, and the H9 viruses had between 86.9% and 95.4% identity. The phylogenetic relationships among the viruses of the H4, H6, and H9 subtype are shown in Figure 1.

DISCUSSION

Herein, we describe the biologic characterization of H4, H6, and H9 LPAI wild bird virus isolates in SPF chickens and commercial turkeys. The objective of this research was to characterize LPAI wild bird isolates through pathogenicity, virus shedding, and serologic

Table 6. Average severity of microscopic lesions found in body organs and tissues of LPAI virus-infected turkeys ($n = 3$) at 3 days PI.

Lesion[A]	Virus[B] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Nasal cavity, trachea, and lung																
Catarrhal rhinitis	–	–	–	–	++	–	+	++	++	++	++	–	++	++	++	++
Lymphocytic rhinitis	++	+	+	+	–	+	+	+	++	++	+	–	++	++	+++	++
Catarrhal tracheitis	+	–	–	–	–	–	+	++	++	+	–	–	–	–	–	++
Heterophilic tracheitis	–	+	–	–	–	–	+	+	+	+	–	–	–	–	–	++
BALT hyperplasia	–	–	–	–	+	–	–	+++	–	+	–	–	–	–	–	+
Heart, breast, and thigh muscle																
Lymphocytic endocarditis	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–	+
Degenerative myopathy	–	–	–	–	+	–	+	+	+	–	++	–	–	–	+	+
Brain	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gastrointestinal tract																
Enteritis	+/++	+	+	+	+	–	+	–	–	+	–	+	++	–	++	+
Typhlitis	–	–	+	–	+	+	–	–	–	–	+	–	++	–	++	+
Enteric necrosis	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
GALT hyperplasia	+	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++
Liver and biliary structures																
Microvesicular hepatopathy	++	+/++	+/++	+ /++	++	–	–	–	–	+	++	–	++	–	–	++
Lipidosis	–	–	–	–	++	–	–	–	–	–	–	–	++	–	–	++
Pancreas	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Spleen																
Histiocytosis	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Bursa and thymus																
Follicle atrophy	+	+	–	+	–	+	–	–	+	+	++	+	++	+	++	++
Lymphoid depletion	–	+	+	–	+/++	+	–	–	+	+	+	+	++	+	++	++
Adrenal gland	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Kidney and reproductive organs																
Interstitial nephritis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[A] – = no lesions; ± = minimal; + = mild; ++ = moderate; +++ = severe.

[B] 1 = MN 00-530, 2 = MN 99-253, 3 = AI 03-114, 4 = LA B156, 5 = TX 02-260, 6 = AI 02-749, 7 = LA 69B, 8 = MN 00-38, 9 = AI 02-1070, 10 = MN 99-263, 11 = AI 01-124, 12 = MN 99-198, 13 = AI 01-1407, 14 = AI 03-452, 15 = LA 240B, 16 = MN 98-232.

response studies. Clinical signs of AI infection can vary based on virus strain, host species, sex, age, immune status, concurrent infections, and environmental factors (27). Clinical signs observed in our study are consistent with previously reported signs (23,27) and ranged from no clinical signs to moderate depression.

Avian influenza viruses are transmitted through excretion of the virus from the nares, mouth, conjunctiva, and cloaca of infected birds (27,30). Oropharynx and cloacal swabs were most often used to isolate and detect the virus (28). Our data shows that SPF chickens were generally found to be shedding more virus from the oropharynx than were commercial turkeys (Tables 2 and 4). This finding is in contrast with the study conducted by Tumpey *et al.* (30), wherein the level of infectious virus recovered from the oropharynx of commercial turkeys was 20- to 158-fold higher than that detected from the same anatomical site in SPF chickens. However, this study examined a poultry-adapted H7N2 virus that had been circulating in live bird markets in the United States since 1994. In this study, low or undetectable viral shedding from the cloaca was observed in both species at days 2, 4, and 7 PI. This may imply that these LPAI viruses replicate more efficiently in the respiratory tract than in the gastrointestinal tract, or that our route of challenge favored respiratory infection (30). The presence of PCR inhibitors in the C swab samples can interfere with optimal virus detection (25). It is possible that inhibitors may vary between chickens and turkeys, and this could have affected the outcome of the virus-shedding data.

Microscopic lesions usually found following LPAI infection in poultry are mostly confined to the respiratory and gastrointestinal tract, some of which include lymphocytic tracheitis, bronchitis, and pancreatitis (27). Our findings are consistent regarding the presence of these lesions. In our study, we examined tissues for microscopic lesions at day 3 PI so that these results could eventually be compared with similar ongoing studies with different AIV subtypes in other laboratories. It is possible that the lesions observed could have become more severe, or could have been resolved with time. Microscopic results in both species at day 3 PI showed the predominance of lesions in the respiratory and gastrointestinal tract (Tables 5 and 6), a finding that is consistent with the fact that these viruses are of low pathogenicity. In chickens, OP shedding strongly correlated with the lesions found in the upper respiratory tract, lesions which were mostly seen in the nasal cavity (rhinitis) and tracheal sections (tracheitis). However, viruses such as AI 01-1407, AI 03-452, LA 240B, and MN 98-232 are notable exceptions, in that mild to severe catarrhal rhinitis was present but there was no OP shedding detected in chickens at all times of swab collection. On the other hand, OP shedding at day 2 PI was detected only in two out of five viruses (MN 00-530 and AI 02-749) that caused lesions in the lower respiratory tract (lung). Shedding of other viruses from the

Table 7. Average ELISA and HI test results for sera collected from LPAI virus-infected chickens.

	Chickens						Turkeys					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
Virus	ELISA[A]	HI[B]	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI
MN 00-530	0.136[C]	9.33	0.557	70	0.466	69.67	0.133[D]	≤2.00	0.637	39.43	1.23	123.43
MN 99-253	0	≤2.00	0	≤2.00	0.039	2.33	0.752	66.57	1.28	103.43	0.299	9.71
AI 03-114	0	≤2.00	0	≤2.00	0.046	≤2.00	0.221	3.43	0.399	28.86	0.281	6.86
LA B156	0	≤2.00	0.045	≤2.00	0.060	≤2.00	0.699	52.00	1.46	93.71	0.287	7.43
TX 02-260	0.011	≤2.00	0.045	≤2.00	0.090	≤2.00	0.378	20.57	0.476	38.28	0.479	24.86
AI 02-749	0	≤2.00	0.101	7	0.027	2.34	0.347	22.29	1.119	105.71	1.402	137.71
LA 69B	0.136	27.67	0.461	33.34	0.281	37.67	0.302	3.71	0.148	20.00	0.217	13.14
MN 00-38	0.031	≤2.00	0.027	≤2.00	0.127	23	0.186	2.28	0.034	≤2.00	0.026	≤2.00
AI 02-1070	0.080	2.67	0.134	≤2.00	0.015	≤2.00	0.640	47.43	0.498	51.14	0.474	35.14
MN 99-263	0.532	37.67	0.533	47.67	0.149	13	0.724	58.4	0.620	34.00	0.539	44.00
AI 01-124	0.086	2.33	0.331	44.33	0.045	2.33	0.546	44.28	1.12	99.43	1.09	102.86
MN 99-198	0.492	43.71	0.469	89.14	0.547	69.71	0.135	2.29	0.676	151.14	0.206	19.71
AI 01-1407	0.005	≤2.00	0.102	≤2.00	0.023	≤2.00	0.068	≤2.00	0.124	≤2.00	0.011	≤2.00
AI 03-452	0.014	≤2.00	0.109	3.00	0.008	≤2.00	0.336	38.57	0.382	10.57	0.467	14.28
LA 240B	0.083	≤2.00	0.084	≤2.00	0.085	8.28	0.111	2.28	0.695	38.28	0.524	31.14
MN98-232	0.002	≤2.00	0.001	≤2.00	0	≤2.00	0.053	2.33	0.213	5.00	0.090	2.33

[A]ELISA = enzyme-linked immunosorbent assay, S/P ratios (values greater than 0.5 are considered positive).
[B]HI = hemagglutination inhibition, each serum sample tested two times (titers greater than 32 are positive for the test).
[C]For the chicken data, each number represents an average of seven samples, except for; MN 99-198, AI 01-1407, and AI 03-452 (days 14 and 21) = average of six samples for each day.
[D]For the turkey data, each number represents an average of seven samples, except for: MN 99-263 turkey data day 7 = average of five samples; day 14 and 21= average of four samples for each day; MN98-232 turkey data= average of six samples for each day.

oropharynx was later detected during the course of infection, as seen in Table 4.

Virus-induced changes in the gastrointestinal tract of chickens in this study include cecal tonsil micro-hemorrhages, GALT hyperplasia, and typhlitis; these were the three most common gastrointestinal lesions due to viral activity (e.g., multiplication and cell damage; Tables 5 and 6). Virus shedding was not detected in most C swab samples at day 2 PI. It is possible that infection occurred, but the virus was not shed, or shedding was below the level of sensitivity for the detection method utilized in this study.

The microscopic lesions in turkeys suggest that this species had fewer lesions in the respiratory tract than did chickens, but had more in the gastrointestinal tract than did chickens (Tables 5 and 6). Turkeys had more microscopic lesions in the respiratory tract than in the gastrointestinal tract, which correlates with the OP and C viral shedding data for turkeys at day 2 PI, wherein there was more viral shedding in the OP samples than in C samples (Table 4). The presence of PCR inhibitors (25) in C swab samples may have played a role in the results of viral shedding. Microvesicular hepatopathy, observed for some of the groups, is most likely due to stress which, in this case, may have come from virus infection. Because no systemic infection was expected with the LPAI viruses, tissue samples were not collected for virus titration. However, microscopic lesions were observed in internal organs including the heart, liver, spleen, and thymus, indicating possible virus replication in those tissues. These findings imply that commercial turkeys are susceptible to the LPAI wild bird isolates used in this study.

The first sign of LPAI infection in domestic poultry is often seroconversion, which may be the only evidence of infection (i.e., no clinical signs present) with some subtypes of LPAI (4). We used the ELISA test rather than the agar gel immunodiffusion test, used in many diagnostic laboratories, because we found it to be easier and cheaper given the large number of serum samples generated in this study. In addition, we confirmed our results for all of the serum samples using the HI test. The serologic data in our study showed that commercial turkeys seroconverted to the majority of viruses tested (13 out of 16), whereas only four viruses induced seroconversion in SPF chickens in both tests. This indicates that turkeys are more susceptible to AI infection than are chickens because seroconversion can only occur following infection (4). Although this study did not include an evaluation of the transmission of these viruses among birds, based on the no, or low, titers of virus being shed, transmission among birds would be unlikely without some adaptation of the virus to the host resulting in higher amounts of virus being shed into the environment (29).

The HA1 region of the HA gene is one of the primary genetic components of AIV involved in host restriction, pathogenicity, and immunogenicity and can provide phylogenetic context for the isolates. Therefore, the HA1 region of each isolate was sequenced to provide additional characterization. Sequence data revealed that two isolates were different subtypes than what was originally identified by the HI assay. This discrepancy is seen occasionally because steric inhibition can cause nonspecific HI activity, or because antigenic variation within a subtype can affect antibody binding (reviewed in [17]). Alternatively, the samples could have been a mixed infection at the time of subtyping, or the isolates may have been mistakenly subtyped for a variety of reasons. It should be noted that the HI test conducted on sera from birds infected with the H1 (AI 02-749) and H13 (AI 01-1407) viruses utilized H9 and H4 antigen, respectively. Thus, the results may not reflect accurate HI antibody titers in those birds; however, the ELISA data did correlate with the HI results. Regardless of subtype, all isolates used in these studies were genetically North American wild bird viruses. Moreover, within the H4, H6, and H9 subtypes, there was genetic variability that was representative of all the major North American clades of each respective subtype.

Although the ability of H4, H6, and H9 wild bird viruses to adapt to domestic poultry varies between individual viruses, and would probably require repeated passages to adapt and efficiently transmit in these new host populations, these viruses are often detected in

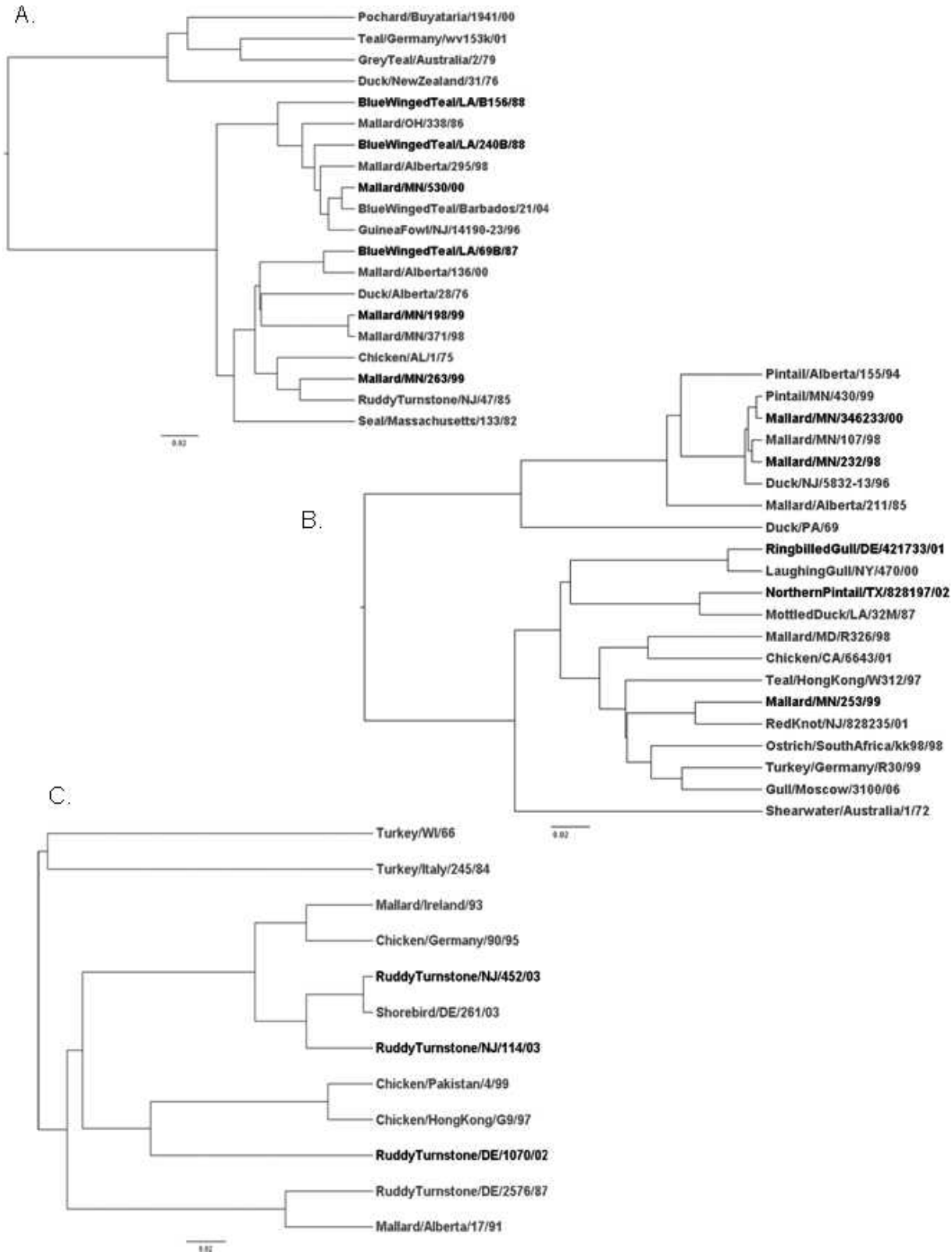

Fig. 1. Phylogenetic trees of the HA1 region of the HA genes of H4 (a), H6 (b), and H9 (c) AI isolates utilized in this study and selected reference isolates. Trees were constructed with BEAST software V. 1.4.8. Isolates utilized in these studies are shown in bold-face type.

domestic poultry populations (2,20). In addition, there is evidence of subsequent human infection with H9 AIV (15). Our results, based on a single direct challenge with LPAI wild bird isolates, suggests that most exposures in chickens and turkeys likely result in little or no transmission. It is likely that the transmission from wild birds to poultry occurs more commonly than currently believed, but may go unrecognized because, in many cases, the virus replicates poorly and fails to cause seroconversion. It is possible that virus

adaptation and subsequent transmission among domestic chickens and turkeys may be enhanced under conditions where domestic ducks can provide a resource for repeated challenge.

In summary, some of the LPAI viruses of wild bird origin examined in this study can infect, and be shed, by chickens and turkeys without causing overt clinical disease or mortality. Moreover, these viruses did replicate in poultry, but induced mild lesions and poor seroconversion. These findings imply that some LPAI viruses of wild bird origin would be difficult to detect in commercial chickens and turkeys and may not be a significant disease threat unless some adaptation of the viruses occurred.

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